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Protein-protein interactions are critical to almost every cellular process. Disruption of these interactions would effectively interfere with the cell's functions and its ability to grow and divide normally.

The Rad51 and Rad52 proteins are important proteins involved in DNA repair. Rad51 acts as a hexamer binding single-stranded DNA to drive strand exchange during homologous recombination. By blocking Rad51 from multimerization we can theoretically disrupt homologous recombination, and thus decrease the efficacy of DNA repair. Deficiency in DNA damage repair will sensitize cells to DNA damaging agents and thus such tumors can be effectively treated with a lower dose of chemotherapeutic agents/radiation.

Short peptides of a few amino acids (5-10) have been shown to be enough to destabilize protein-protein interactions. Thus a library of random combinatorial peptides of sufficient complexity will in theory have an inhibitory molecule for any protein-protein interaction. This project attempts to isolate peptides that inhibit Rad 51 from multimerisation to be used as a chemosensitizing agent during chemo/radiotherapy using a modified Yeast two hybrid screen called the reverse two-hybrid system.

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Identification of novel inhibitory peptides of protein-protein interactions involved in DNA Repair as potential drugs in Breast Cancer Treatment

(4) Introduction:

Tumorigenesis is the result of multiple genetic changes. Although cells are subject to a multitude of environmental and chemical factors, the cell's robust repair machinery is able to repair most of the damage and, if not, at least program the cell to undergo apoptosis thereby preventing uncontrolled proliferation. However mutations in these check-point genes which diminish the cell's ability to do either of these functions may lead to increased susceptibility to neoplasias. Hereditary Nonpolyposis colorectal cancer syndrome (HNPCC) is an example of such an inherited mutation causing increased susceptibility to cancer (2).

Homologous recombination is one of the more important repair pathways that guards against tumorigenesis. While its prominent function is the exchange of information during meiosis, its has been shown to be a key pathway in DNA repair in bacteria (3) and yeast (5). In bacteria Rec A is the protein that drives homologous recombination. Rec A acts as a hexamer, binding single-stranded DNA and driving strand exchange. It has been shown that Rec A is a critical component of the SOS response to ionizing radiation (3). In *Saccharomyces cerevesiae* The Rad 51 and Rad 52 proteins have been identified as important players in this pathway. Rad 51 is the yeast homologue of the bacterial Rec A protein. A mammalian homologue of Rad 51, with high homology to the bacterial Rec A protein, has also been cloned. The high degree of conservation between prokaryotes and high order eukaryotes suggests the importance of this pathway for the cell.

BRCA1 and BRCA2 are tumor suppressor genes identified in breast cancer. Although the primary function of these genes has not been fully elucidated, they are thought to have a role in DNA damage repair. BRCA1 has 1863 amino acids and BRCA2 3418 amino acids. There are no homologues for either protein in yeast. A wide spectrum of both mis sense and truncation mutations have been identified in these genes. It has also been found that BRCA2 interacts with Rad51. Both BRCA1 and BRCA2 knockouts are embyonic lethal in mouse (4). BRCA2 has eight repeating motifs named BRCA repeats (BRCT). Both proteins have been shown to be cell cycle regulated, peaking during the S phase of the cell cycle. BRCA2 has also been shown to bind Rad51 underlying the importance of these proteins in DNA repair.

(5) Body :

Protein-protein interactions are critical to almost every cellular process from cellular macrostructures to enzyme complexes and signal transduction. As such, disruption of these interactions will provide a mechanism of deregulation of the respective pathways and hence a molecular target for drugs.

Most therapeutic agents for breast cancer function by either directly (ionizing radiation) or indirectly (topoisomerase inhibitors) causing DNA damage. The problem with these agents is the generalized toxicity of the treatment. Therefore any agent that can specifically target the breast tumor can be used to sensitize the tumor alone to the DNA damaging agent.

Mice lacking BRCA2 and Rad51 have a Rad51-associated hypersensitivity to gamma radiation (7). However BRCA2 deficient breast epithelial cells can survive with an unstable genome and thus proliferate, especially if they have another genomic alteration associated with malignancy. We think that loss of function of BRCA2 might increase genomic instability due to increased or error prone homologous recombination mediated by Rad51. In this context inhibition of Rad51 function may decrease the proliferative capacity of these cells. Also BRCA1 and BRCA2 mutations are estimated to be responsible in 80 % of inherited cases of breast cancer and more than 95 % of inherited ovarian cancers. This strong correlation provides an ideal molecular target for treatment of these cases. While normal cells have the rates and activities of the homologous recombination (HR) machinery in check through tumor suppressors BRCA1 and 2, tumor cells which are BRCA null will have deregulated HR pathway. Thus inhibition of homologous recombination in these cells will preferentially sensitize them to treatment.

The DNA repair pathway is thus an obvious target for an agent that disrupts protein-protein interactions. One of the more important pathways of DNA repair is through homologous recombination. By blocking Rad51 from multimerization we can theoretically disrupt homologous recombination, and thus decrease the efficacy of DNA repair. Deficiency in DNA damage repair will sensitize cells to DNA damaging agents and thus such tumors can be effectively treated with a lower dose of chemotherapeutic agents/radiation.

Isolation of Inhibitory Peptides

Short peptides of a few amino acids (5-10) have been shown to be sufficient to destabilize protein-protein interactions. Thus a library of random combinatorial peptides of sufficient complexity will in theory have an inhibitory molecule for any protein-protein interaction. We proposed to use the reverse two-hybrid system to isolate inhibitory peptides (8). The system is a modification of the conventional two-hybrid system but selects for a protein that will destabilize a protein protein interaction and was developed by Mark Vidal (8). This is done by introducing a Ura3 gene under the Gal 4 promoter as one of the reporter genes. Basal expression of Ura3 is inhibited by engineering an Upstream Repressing Sequence (URS1) of Spo13 upstream of Ura3 (2). Ura3 encodes Orotidine 5'phosphate decarboxylase, used in the biosynthesis of Uracil. Ura3 can also catalyze the conversion of 5 Fluoro orotic acid (FOA) into a toxic product, 5 fluorouracil. Accordingly, if we transform this host strain with plasmids harboring

genes whose products are known to interact (as fusions with Gal 4 binding domain and Gal 4-activation domain) the yeast will be able to grow in a media lacking Uracil. However the strain will be sensitive to the presence of FOA (Fig. 4). Thus if we now introduce a library of random peptides one of which may potentially inhibit this interaction into this cell, we may suppress the expression of Ura 3. A cell harboring such a peptide will be Ura^{-} FOA^{R} and thus can be selected for.

The strain we proposed to use was MaV103 (MATalpha leu2-3, 112 trp1--901 his3D200 ade2-101 gal4D gal80D SPAL10::URA3 GAL1::lac Z Gal1::his3@LYS2 can1R cyh2 R) (8). The host strain has 3 stably integrated Gal4p-inducible genes: SPAL::URA3, integrated at URA3, Gal1::HIS3, integrated at LYS2, and GAL1::LacZ integrated at an unknown locus.

Double strand breaks occur both spontaneously as a result of DNA metabolism as well due to exogenous DNA damaging agents such as UV, ionizing radiation and chemotherapeutic agents. Homologous recombination is a major pathway in repairing these double strand breaks (DSB). Disruption of this pathway by targeting Rad 51 protein will sensitize cells to chemotherapeutic agents and thus will reduce dosage requirements considerably. The overall objective of this proposal was to develop a novel genetic screen to identify inhibitory peptides of molecular interactions of Rad 51 to be used as a chemosensitizing agent in cancer therapy.

Specifically the plan was to:

1. To construct a random combinatorial library of 15 amino acid peptides consisting of all possible combinations of the 20 available amino acids.
2. To set up a system for the selection of inhibitors of Rad 51 self interaction by establishing yeast two hybrid system with Rad51-Gal 4 DNA binding domain and Rad51- Gal4 Activation Domain fusions cloned in *S. cerevisiae* strain MV103.
3. To transform this library into the established system and identify peptide inhibitors of Rad 51 protein in the context of a reverse two hybrid system.
4. To characterize these inhibitory peptides and test their ability to sensitize cells to DNA damage.

Methodology:

1. Construction of a combinatorial Library:

A random DNA library coding for all possible combinations of 15 amino-acid peptides was to be constructed. The vector pHANLS we used was derived from the commercially available pGAD vector. The Gal4 activation domain was removed and a nuclear localization signal (NLS) was added upstream of the multiple cloning site (MCS) at the *Nco I* restriction site. A HA tag was added immediately downstream of the NLS. The vector was constructed by Dr. Zhiyuan Shen presently at U. New Mexico at Albuquerque.

We encountered a variety of unforeseen problems at this stage, so we tried three different approaches (Fig.1).

Approach 1:

A template for the library (Ran TMP) containing N_{45} flanked by 15 bases on either side for primer binding and restriction was custom synthesised from GibcoBRL. The library was then synthesised by PCR using Ran TMP as template, precipitated using ethanol and then digested at the introduced *EcoR I* and *BamH I* sites and cloned into pHANLS vector. However this approach did not work very well and the tranformation efficiency was always low (the maximum complexity we could get was in the order of 10^3).

Approach 2:

During a meeting on Phage Display technologies (MIT, April 7-9, 2001) we had an oppurtunity to talk with Dr. Jonathan Blum (Harvard Medical School). He had done some work using combinatorial DNA libraries to isolate peptides with antibacterial action (1). We learned that he had also tried the approach described earlier and had encountered the same problems as us. He suggested we use Klenow fill-in to generate our library. We then used the same template as before but instead of PCR amplifying the library we used Klenow enzyme and a single primer at the 3' end. The product was then purified as before and the ligation and cloning was carried out. However we encountered new problems at this step. The small insert size (47 bases after restriction) provided a lot of background with no or multiple inserts. So we chose approach 3.

Approach 3:

A 77 nucleotide DNA fragment custom ordered from GIBCO-BRL as the template for the library. The 3' end of the oligonucleotide is complementary to the vector pHANLS and has 15 repeats of NNK ('N' stands for any nucleotide and 'K' for the base 'A' or 'T'). Each 'NNK' will serve as a codon for amino acid synthesis. Using this oligo as the 3' primer and a 5' primer from the upstream portion of pHANLS we can generate a double stranded DNA fragment with the library at the 3' end. This approach addressed a variety of problems encountered in the previous approaches:

- Accomodating for the degeneracy of the codon usage allowed us to reduce the complexity required to cover the entire sample space by an order of 2^{15}.
- Incorporating the library as a primer will prevent the amplification bias introduced by PCR.
- This method also allowed us to synthesize the library as a much larger fragment and made it easier to handle and manipulate the library DNA during construction.

Using a restriction site incorporated in oligo primers we were able to directionally clone the library into the vector. Scaling up at this level will give us a library complexity of 10^6. We are presently fine-tuning the process to increase the complexity of the library to 10^8.

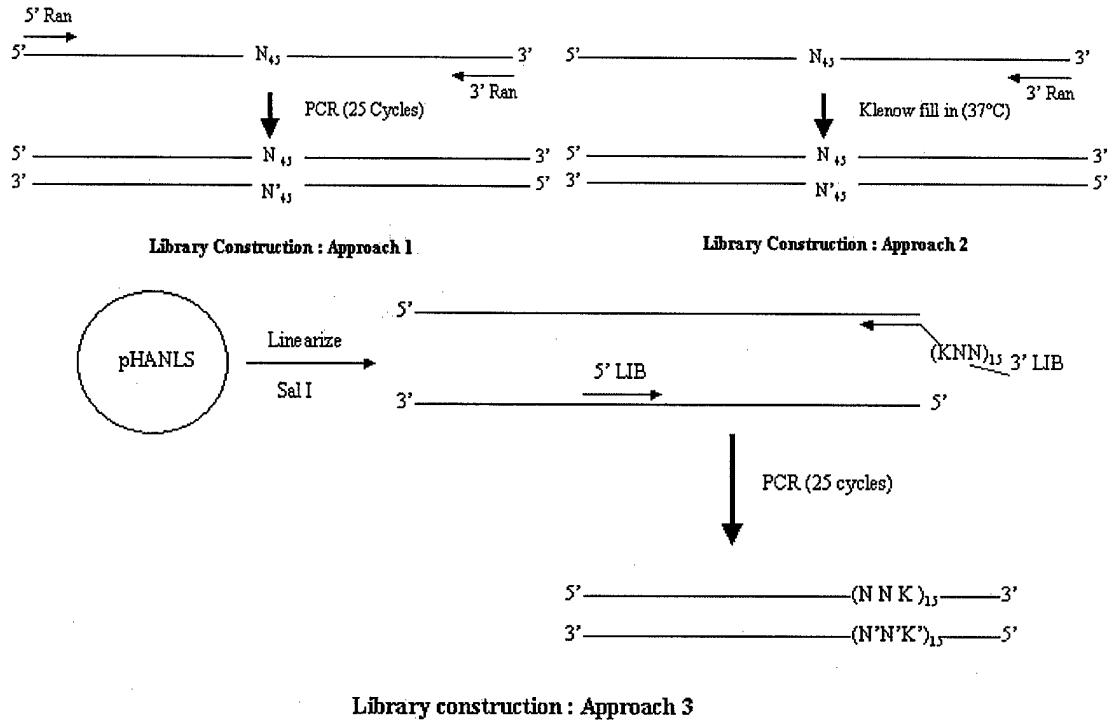
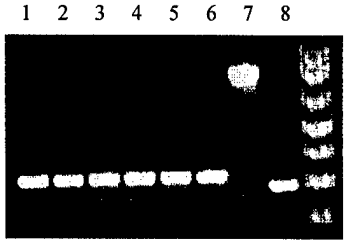


Fig. 1: Library construction.

Fig. 2: Colony PCR was performed using primers in the vector pHANLS to look for library inserts. A vector with no insert gave a band of length ~280bp (Lane 8) while a plasmid harboring a random nucleotide insert gave a band of length ~330bp(Lanes 1-6). Lane 7 is a positive control (pHANLS harboring a 520bp insert).

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pHANLS/1 CCGGGGATCCGTCGACCT AAAGCCCCCTCCATCCCCGCAAATTCTGCCACC.ACCACCGCACA  AGATCTATGAATCG
pHANLS/2 CCGGGGATCCGTCGACCT CCCAACGATAATTCCTACCCCCCGCAACATACTACCTCCTCACA   AGATCTATGAATCG
pHANLS/3 CCGGGGATCCGTCGACCT GCCCAACGATAATTCCTACCCCCCGCAACATACTACCTCCTCACA  AGATCTATGAATCG
pHANLS/4 CCGGGGATCCGTCGACCT CTCCGAACCTTCCCCCTCCACCCCTGCCCCTCCGACGCCCCCATA  AGATCTATGAATCG
pHANLS/5 CCGGGGATCCGTCGACCT TTCTCCCCCACCTCCCCCAGCTCCAAACCATGAAACCCAGTCACC  AGATCTATGAATCG
pHANLS/7 CCGGGGATCCGTCGACCT CTCCCCCACCTCAGCCTCACCCCCCCCGGATGCACCTCCCTCTCC  AGATCTATGAATCG
pHANLS   CCGGGGATCCGTCGACCT ..........................................  AGATCTATGAATCG
```

Fig. 3: Randomness of library as shown by sequencing a few colonies: 45 bp random insert flanked by vector sequences. Last sequence is empty vector.

2. The Reverse Two hybrid Set up:

The next part of our plan was to set up a two hybrid system of Rad 51 interaction in *S. cerevisiae*. Rad 51 fusions were constructed with the Gal 4 activation domain (Gal4 AD) and the Gal4 DNA binding domain (Gal4 BD). The Rad 51 cDNA was obtained from Dr. Zhiyuan Shen (U. New Mexico). The insert was PCR amplified and cloned into the pGDBMAD vector, also provided by Dr. Zhiyuan Shen. This vector is derived from the commonly used pBRIDGE and has two multiple cloning sites for fusion with the Gal4 AD and Gal4 DB and has a Tryptophan selection marker.

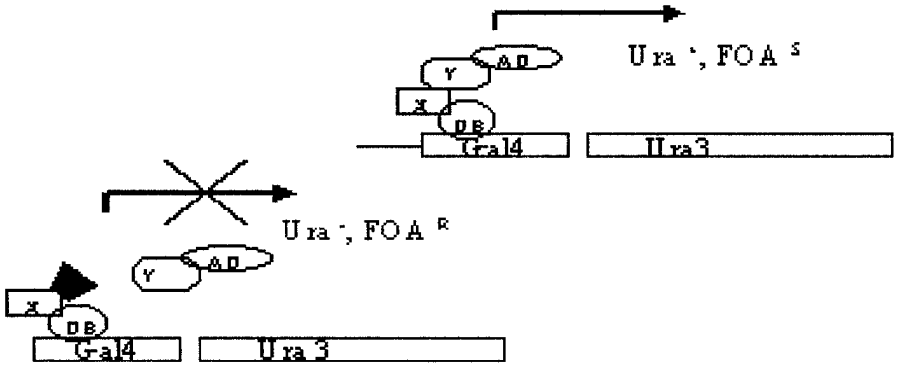

Fig. 4: Reporter Gene Function.

Three plasmids were thus constructed:

PGDBMAD/AD51- with Gal4 AD-Rad51 fusion

pGDBMAD/DB51- with Gal4DB-Rad51 fusion

pGDBMAD/MAD51- with Gal4 AD-Rad51 and Gal4DB-Rad51 fusions

The vectors were then transformed into MV103 and the transformants selected on Trp⁻ plates. The clones obtained were then assayed for Lac Z activity (Fig. 5).

Fig. 5: Lac Z assay. 3 clones from each transformation with the respective vectors (pGDBMAD, pMAD51, pDB51 and pAD51) were grown overnight. A colony lift Lac Z assay was then performed and color development was observed after 3 h. Only clones that harbored pMAD51 tested positive.

Determination of minimum 5 FOA concentration:

Three clones from each transformation were taken and grown on Trp$^-$ plates containing 0%, 0.05%, 0.1%, 0.16% and 0.3% 5FOA by weight. Clones harboring pMAD51 are sensitive to 5FOA and are killed between 0.05% and 0.1% FOA. In the next step the clones were plated on plates containing 0%, 0.02%, 0.04%, 0.06% & 0.08% FOA. Minimum FOA concentration for cell death in MV103/pMAD51 was 0.06% (Fig. 6).

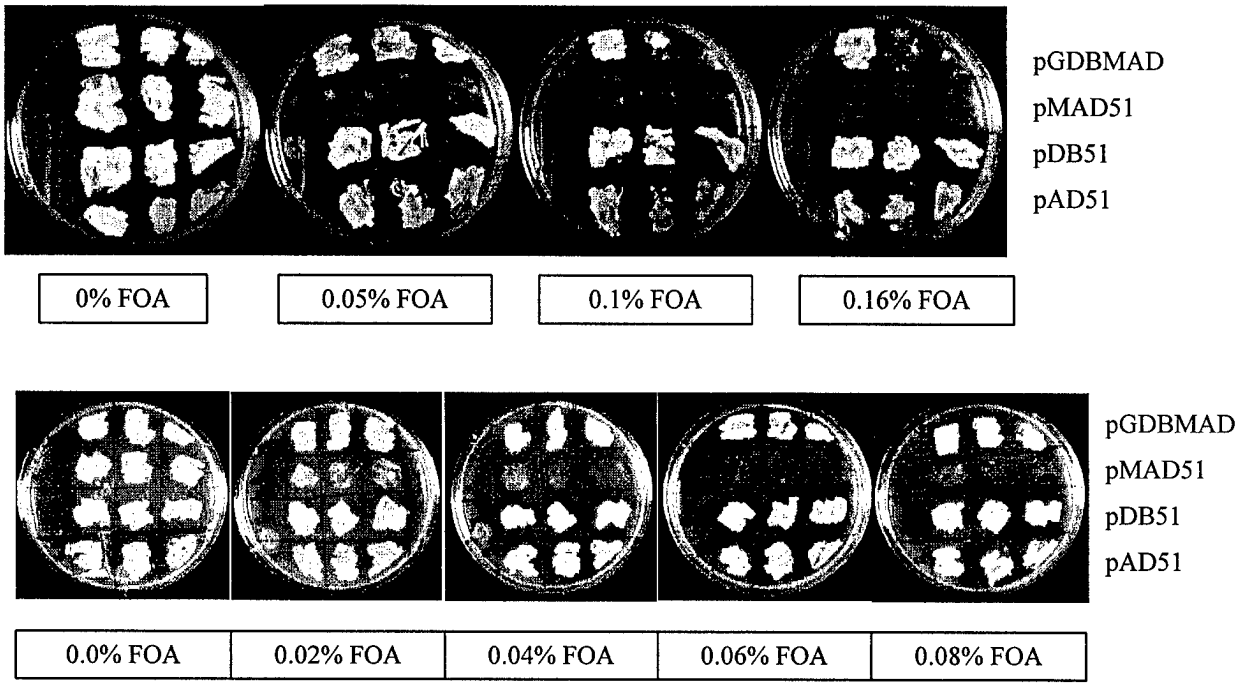

Fig. 6: Determination of minimum FOA concentration.

Future Directions:

Screening for Inhibitory peptides:

Once we have a library of sufficient complexity (> 10^7) it will be transformed into the two hybrid set up detailed in (2) and selected for inhibitors of Rad 51 interaction on Ura$^+$ FOA$^+$ plates. The clones harboring the plasmid for inhibitory peptides will then be isolated and the corresponding plasmid extracted.

Anticipated problems: The genetic screen we propose to use is not designed to specifically select for peptide inhibitors. The screen may isolate also antisense RNA. We propose to use an additional step to determine if the plasmid isolated from the screen codes for antisense RNA or peptides. This will be done by sub-cloning the construct into a vector PHANLS/stop which has an inbuilt stop codon following the HA tag. If the construct codes for antisense RNA then the new construct will still select in the Ura$^+$

FOA^+ media while it will not if the construct codes for a peptide. We also plan to do in viro pull down experiments using GST tagged Rad 51 to confirm disruption of Rad 51 interactions by the peptide isolated from the screen.

Characterization of inhibitory peptides:

The plasmids that are positive for the selection will be isolated and sequenced. The pHANLS vector has a HA tag attached at its N terminus of the insert. The peptides will be expressed and purified using the HA tag and used in in vitro studies to look at their potency to disrupt Rad51 self-association. Also cell survival assays will be performed to determine if the peptides are active in cell culture and if disruption of the HR pathway through disassociation of Rad 51 sensitizes cells to double strand breaks.

We will use the *I-SceI* system previously described by Pierce *at al.*(8) to investigate the efficiency of Rad 51 mediated homologous recombination in cells expressing inhibitory peptides(5). This system which uses a rare cutting endonuclease to induce DSB at specific sites in cell culture. The cells are then trasfected with a retroviral vector encoding EGFP (under a constitutive promoter) with a *I-Sce I* restriction site in the coding region and and another vector harboring a promoterless EGFP construct. The eficiency of homologous recombination is then studied by assaying for EGFP reconstitution or cell survival.

(6) Key Research Accomplishments:

- A random DNA library of complexity in the order of 10^6 encoding 15 amino-acid peptides was synthesized.
- A system to select inhibitors of Rad51 self association in the context of a Reverse Two hybrid System was set up.

(7) Reportable Outcomes:

None

(8) Conclusions:

We now have the set up ready for screening inhibitory peptides against Rad51 self association. We have determined the minimum FOA concentration required for the screening at 0.06%. We also have created a combinatorial DNA library coding for 15 aminoacid peptides at a complexity of about 10^6. We are in the process of increasing the library complexity to about 10^8. Once we have a library of such complexity we can start screening for inhibitory peptides.

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